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**14. ABSTRACT**

The glucocorticoid receptor (GR) is a hormone-dependent transcription factor involved in the regulation of a wide range of metabolic anddevelopmental processes by controlling the expression of target genes in a hormone- and cell-specific manner. However, the expressionand activity of GR in normal and malignant prostate growth is unclear. We have recently developed a GR phosphorylation site specificantibody to serine 211 of human GR (GR-S211-P) and found a strict correlation between phosphorylation of GR at this site and receptortranscriptional activity. Thus, GR phosphorylation at S211 is a surrogate marker for the ligand-bound and transcriptionally active form ofGR in vivo. Using this antibody to survey GR phosphorylation in human tissues by immuohistochemistry, we came across the remarkablefinding that ligand bound and transcriptionally active phospho-GR is present in the stroma and epithelium of normal prostate tissue,including basal and luminal epithelial cells. This was not the case for other tissues examined and suggests that the prostate is becontinually exposed to glucocorticoids, such that GR is actively signaling in the prostate. T he experiments described in this proposal aredesigned to elucidate t he role of GR in prostate cell growth.

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***Introduction***

Glucocorticoids, via the glucocorticoid receptor (GR), regulate the expression of target genes in a hormone- and cell-specific manner to suppress cell growth. Clinically, glucocorticoids are used as a last resort to treat hormone refractory prostate cancer and have been shown to have little clinical benefit. Limited data on GR expression in the prostate suggests that GR levels in the epithelium are reduced in cancer relative to normal tissue, suggestive of a role for GR in growth suppression of prostate epithelial cells. However, a direct demonstration that GR regulates prostate cell growth and differentiation has not been shown.

***Body***

Because GR typically stop cells from growing, we hypothesized that GR is regulating genes that restrict prostate epithelial cell growth and that its loss would promote prostate cellular proliferation and prostate cancer. To test this hypothesis, we propose to characterize the expression of GR and phospho-GR S211P (a surrogate biomarker for the activated GR) and to generate a mouse lacking GR specifically in prostate epithelial cells and examine changes in proliferation and differentiation.

Since a conventional knockout of the GR gene in the mouse is lethal at birth, we needed to produce a "conditional" GR knock out mouse where we could remove GR specifically from prostate epithelial cells and determine the contribution of GR to prostate epithelial cell growth.

***Key research accomplishments***

*Task 1* Characterization of phospho-GR S211 expression in prostate cancer and prostate development

a. Analyze normal prostate tissue as well as prostate cancer samples for alterations in phospho-GR, GR and AR protein expression by immunohistochemistry. (months 1-6)

To examine the role of GR in normal human prostate development, we performed immunohistochemistry on specimens from 14-week fetuses using an affinity-purified GR Ser (P)-211 or an antibody that recognizes GR independent of its phosphorylation state (total GR). Sections through the urogenital sinus region show a cell-free region in the center, which is the lumen of the urethra (Fig 1, *A-C, areas* marked *U*). Adjacent to the lumen are columns of epithelial cells that are surrounded by mesenchymal or stromal tissue (Fig. 1, *areas* marked *str*). Staining with the total GR antibody showed GR protein in the layer(s) of the epithelial cells adjacent to the lumen of the urethra, but not in stromal cells (Fig. 1 *A*). Incubation with phospho-GR antibody however, showed only a subset of epithelial cells in the prostate buds staining (Fig. 1, *D)*. Because GR plays an important anti-proliferative role in most tissues, the samples were also stained with Ki67, a marker of cellular proliferation to determine whether GR or phospho-GR correlates with cellular proliferation (Fig. 1, *B* and *E*). The patterns of Ki67 and phospho-GR staining rarely overlap, indicating that GR Ser-211 is unlikely to be phosphorylated and therefore active in cells that are proliferating.

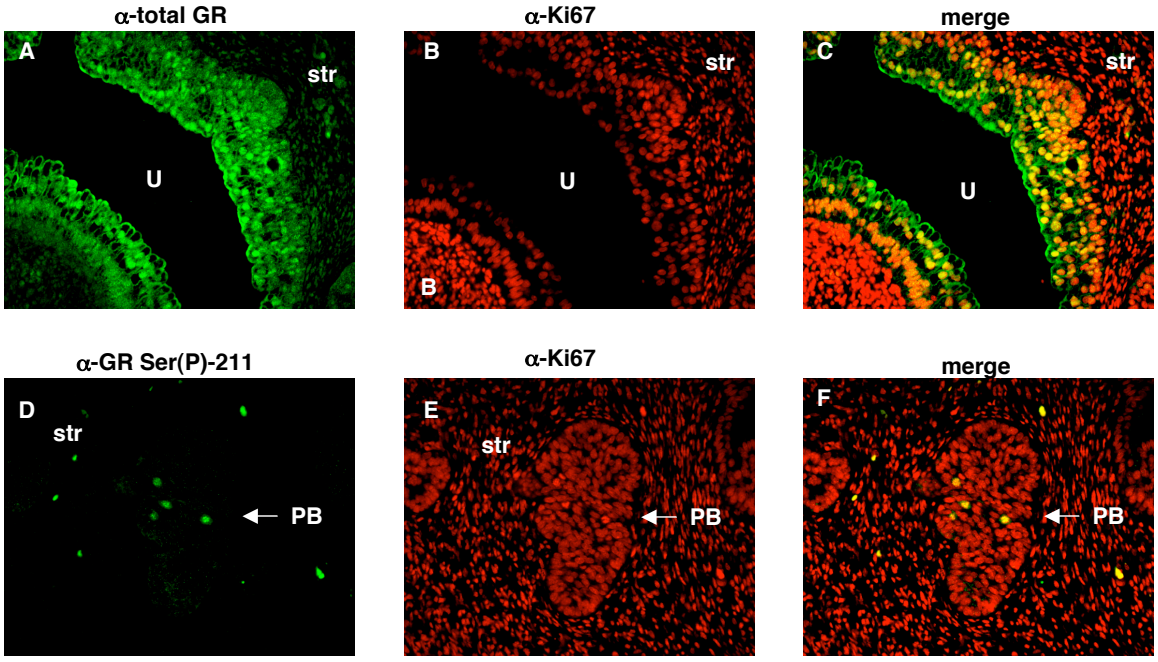


**Figure 1. GR and phospho-GR expression in human prostate development.**

*Panels A-F* show immunohistochemistry via indirect immunofluorescence using paraffin sections of 14-week-old human fetal tissue from the region of the urogenital sinus. The lumen of the urethra (*U*) is indicated for orientation. Tissue samples were incubated with either total GR antibody (*A*) GR Ser (P)-211 antibody (*D*), or the cell proliferation marker Ki67 (*B* and *E*). At 14 weeks total GR was detected in epithelial cells adjacent to the lumen of the urethra but not in the surrounding stroma (*str*) (*A*). GR Ser(P)-211 was present in the nuclei of a subset of epithelial cells in the prostate bud (PB) but not in the surrounding stroma (*D*). Ki67 stained stromal cells and cells in the prostate buds, but not epithelial cells adjacent to the urethra. Immunostaining utilized affinity-purified rabbit polyclonal total GR and phospho-S211GR antibodies and fluorescein isothiocyanate-conjugated secondary antibody (*green*) and mouse monoclonal Ki67 antibody and rhodamine-conjugated secondary antibody (*red*). The merging of the two signals (*D and F*) indicates that GR Ser (P)-211 and Ki67 co-localize in only a small number of cells in the prostate buds.

We also attempted to examine GR and phospho-GR expression in prostate cancer, but due to the poor quality of our prostate cancer tissues specimens, we were unable to obtain reproducible results. Therefore, this part of the task was not completed.

*Tasks 2* To create GR null mice in prostate epithelial cells and assess the consequences of lack of GR expression on prostate development (months 3-18).

a. Produce double transgenic mice with both the probasin-Cre transgene and a conditional floxed GR allele (months 3-12)
b. Characterize GR deficient prostate mutant mice for defects in cellular proliferation (months 6-18)

We have successfully generated a conditional GR knock out mouse where the GR gene can be selectively removed by the Cre recombinase (Figure 2). Prior to embarking on the GR knock out in prostate epithelial cells, we needed to validate our mouse model in a GR expressing cell type where the removal of the receptor had been previously shown to have functional consequences *in vivo*. Therefore, we choose to authenticate our model in T-cells, where the loss of GR in early thymocyte development has been shown to result in severe gastrointestinal inflammation and mortality upon T-cell activation (1).

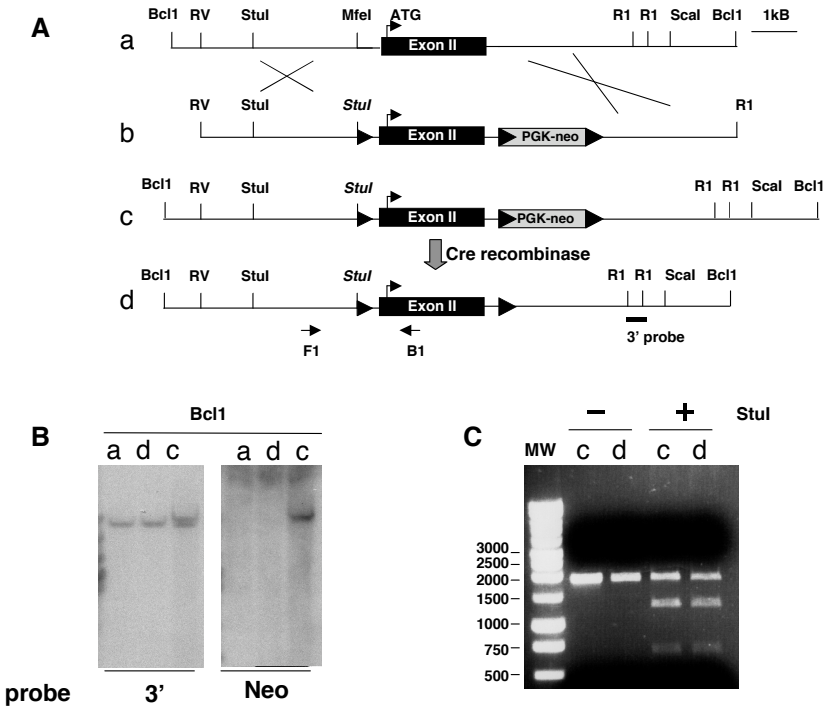


**Figure 2 Generation of a floxed conditional allele of *GR* (*GR*flox).**
(*A*) Schematic representations of a) the wild-type *GR* locus flanking exon II, b) targeting vector, c) recombined locus, and d) the final conditional allele of *GR*(*GR* flox) with the PGK-neo selection cassette removed are shown. *B*) Confirmation of the *GR*flox allele by Southern blot. DNA prepared from ES cell clones was digested Bcl1 and Southern-blotted for hybridization with the 3' and neo probes indicated in *A*. C) Presence of the loxP site in the *GR*flox allele was confirmed by PCR. DNA from ES cells was amplified by PCR using F1 and B1 primers. The PCR products, either uncut (-) or digested with Stu I (+) were resolved on an agarose gel and visualized by ethidium bromide staining.

We were successful in selectively ablating GR from thymocytes and recapitulating the above phenotypes, thus establishing our mouse model as a valuable new tool to analyze GR function *in vivo*. Now that we have a mouse generated a mouse where the GR gene can be excised in a tissue specific manner, we will initiate studies on the consequences of the loss of GR in the prostate using a prostate epithelial cell specific Cre recombinase-expressing mouse (2).

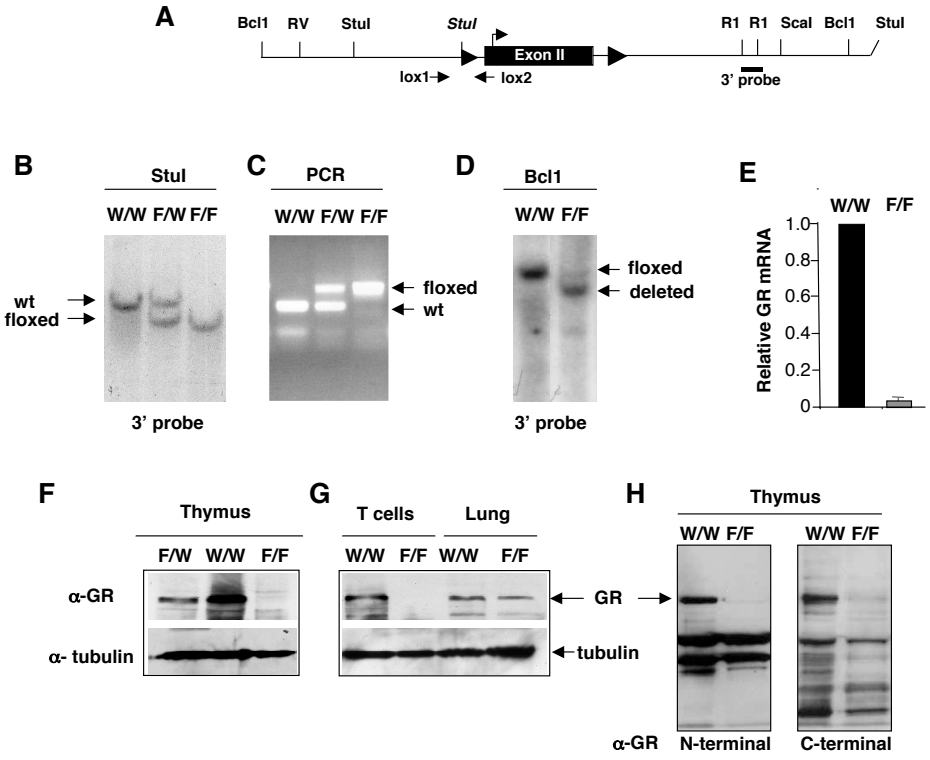


**Figure 3 Deletion of GR in T-cells of *GR*flox mice.**
(*A*) Schematic diagram of the *GR*flox conditional allele. B) Southern analysis of *GR*flox mice. DNA from wild type (W/W), heterozygous (F/W) or homozygous *GR*flox ($GR^{F/F}$) mice was digested with StuI and Southern-blotted for hybridization with the 3' end probe indicated in A. B) Genotyping of *GR*flox mice by PCR. DNA was amplified by PCR using Lox1 and Lox2 primers and the resulting DNA fragments were resolved on an agarose gel and visualized by ethidium bromide staining. D) DNA prepared from the thymus of mice were digested with Bcl1 and Southern-blotted using a 3' probe. (E) mRNA abundance of GR in $CD4^+$ or $CD8^+$ T-cells was assessed by qPCR using 28S RNA as the normalization control. F) Western blot analysis of protein extracted from the whole thymus using an antibody against GR and tubulin. G) Western blot analysis of protein extracted from purified $CD4^+CD8^+$ T-cells or lungs from mice of the indicated genotype using an antibody against GR and tubulin. H) GR protein immunoblot of whole thymus using antibodies to the GR N-terminus (*left panel*) and C-terminus (*right panel*). A similar non-specific pattern of bands is observed in both $CD4/GR^{wt/wt}$ (WT) and $CD4/GR^{f/f}$ (Flox) samples.

***Reportable outcomes***

See attached manuscript.

***Conclusions***

We have analyzed GR and phospho GR expression in human prostate development (Figure 1). Our findings indicate that total GR is expressed in differentiated prostate epithelial cells. In addition, phospho GR S211-P, which represents the active growth suppressive form of GR, is expressed in only a few cells of the proliferating prostatic bud, suggesting that the "activated" form of GR is largely absent in proliferating tissues. This finding is consistent the hypothesis that the function of GR in the prostate is anti-proliferative.

We have also generated a "conditional" GR knock out mouse (Figure 2) and have validated this new mouse model in thymocytes, a known GR target cell, as a prelude to the inactivation of the GR gene in prostate epithelial cells (Figure 3). We will soon be breeding these conditional GR mice with probasin-Cre expressing mice to ablate GR from the prostate in order to analyze the consequence of GR in prostate development. These studies undoubtedly will shed light on the mechanism of growth regulation by GR in the prostate.

***Bibliography***

Ismaili, N., Pineda Torra, I., Shen, Y., Lee M-J. Littman, D.R., Garabedian, M.J. Stage specific T-cell responses in mice lacking the glucocorticoid receptor differentially affect the inflammatory response *(submitted)*

***Personnel receiving pay from the research effort:***

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Michael J. Garabedian, Ph.D.

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***Appendices:***

***See attached manuscript***

# Stage specific T-cell responses in mice lacking the glucocorticoid receptor differentially affect the inflammatory response

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Running title: GR stage-specific imprinting in thymocytes

***Abstract***

We have selectively inactivated the glucocorticoid receptor (GR) in $CD4^+CD8^+$ thymocytes using a Cre–Lox approach. T-cell subsets in the thymus were unchanged between the GR knock out and control mice, indicating that GR is dispensable for T-cell development. However, a small decrease was observed in the number of splenic T-cells lacking GR, suggesting that GR affects T-cell trafficking or survival in the periphery. Surprisingly, and in contrast to a previous report that GR gene inactivation at the earlier $CD4^-CD8^-$ stage of thymocyte development resulted in derepression of COX-2, intestinal inflammation and lethality upon T-cell activation, mice lacking GR at the later $CD4^+CD8^+$ stage displayed none of these phenotypes. Thus, the phenotypic consequences of the GR loss in thymocytes are stage-specific, suggesting that GR imparts an epigenetic memory early in thymocyte development that persists in mature T-cells to limit the inflammatory response. Our findings suggest that in addition to the well-established direct repression of inflammatory gene expression through protein-protein interaction, GR can also antagonize the activation of proinflammatory genes through a novel epigenetic mechanism in thymocytes. This may link the pathophysiology of T-cell mediated inflammatory diseases to defects in GR signaling during specific stages of thymocyte development.

***Introduction***

Glucocorticoids are known to influence immune function and have long been used as anti-inflammatory and immunosuppressive agents (8). Glucocorticoids mediate their immunoregulatory effect by binding to the intracellular glucocorticoid receptor (GR) (31). GR is a transcriptional regulatory protein with three functional domains: an N-terminal transcriptional activation function (AF) 1, a central zinc finger DNA binding domain (DBD) and a C-terminal hormone-binding region that contains a ligand-dependent AF2 (32). In the absence of hormone, the Hsp90-based chaperone complex represses GR regulatory activities (20). Hormone binding relieves this repression and results in a conformational change in the receptor, which promotes DNA binding as well as an association with transcriptional regulatory cofactors to enhance the transcription of target genes. GR also modulates transcription independent of direct DNA recognition via protein-protein interactions (14). Such a "tethering" mechanism is responsible for the repressive effect of GR on transcription factors such as AP1 and NFkB, to suppress the inflammatory response.

It is well established that thymocytes are sensitive to glucocorticoid–mediated apoptosis (2, 25). Thymocytes are divided into different subsets based on the expression of cell surface markers. Thymocytes differentiate from the $CD4^-CD8^-$ double negative (DN) cells to the intermediate $CD4^+CD8^+$ double positive (DP) stage. The majority of the DP cells has T cell antigen receptors (TCR) with sub-threshold affinity for complexes of self-peptides and major histocompatibity complex (MHC) molecules, and undergoes apoptosis or "death by neglect". The remaining DP cells with the ability to recognize self-major histocompatibity complex MHC-II or MHC-I molecules are positively

selected and differentiate respectively into either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) cells. DP cells with high affinity for peptide-MHC and the potential to be self-reactive are eliminated.

Steroidogenic enzymes are present in the thymic epithelium, which may produce glucocorticoids locally to influence thymocyte survival and differentiation (19, 30). Thus, the thymus may represent a unique microenvironment that could allow directed delivery of corticosteroids to thymocytes (2).

High doses of glucocorticoids are known to induce thymocyte apoptosis and this process is inhibited by TCR-mediated activation of the ERK signaling pathway. Conversely, induction of apoptosis by TCR ligation is repressed by glucocorticoids (1, 13). In view of this "mutual antagonism", it was suggested that GR plays a role in thymopoiesis by regulating the threshold of TCR-mediated positive and negative selection (2).

To assess GR function during T-cell development *in vivo*, several groups have manipulated the level of GR in the mouse and analyzed T-cell development with conflicting results (reviewed in (2, 7, 15). Using two independent transgenic mouse models that express an anti-sense GR cDNA (referred to as TKO), two groups reported contradictory results regarding the involvement of GR in T-cell development: while King *et al* reported a 90% reduction of the number of DP thymocytes, Sacedon *et al*, reported a significantly increased thymocyte number in a comparable model (17, 24). The disparity between these studies may reflect differences in the specificity promoters used to drive the anti-sense transgenic expression.

Mice with a disrupted GR gene (GR null mutant, GRKO) die at birth due to defects in lung maturation (4). Total thymocyte number and single positive subsets were unchanged in $GR^{+/-}$ mice, and their ability to undergo negative selection was normal in fetal thymus organ cultures from $GR^{-/-}$ embryos (21). However, embryonic thymocytes isolated from these mice and cultured *ex vivo* were resistant to glucocorticoid-induced apoptosis, implicating GR directly in this process.

In transgenic mice carrying two additional copies of the GR gene, an increased sensitivity to glucocorticoid-induced apoptosis was observed in primary thymocytes (23). Furthermore, to distinguish between the biological functions of GR that require binding of homodimers to DNA versus those that depend on protein-protein interactions, a GR dimerization defective mutant ($GR^{dim/dim}$) was generated (22). The $GR^{dim/dim}$ mice survived to adulthood indicating that receptor DNA binding, as a homodimer is dispensable in early stages of mouse development. Interestingly, thymocytes from these GR dimerization mutants were also resistant to glucocorticoid-induced cell death indicating that receptor dimerization-dependent DNA binding is necessary for glucocorticoid-induced apoptosis of immature T-cells. However, unlike the GRKO mice, where the number of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ cells was reduced, in the $GR^{dim/dim}$ mutant mice, the thymocyte populations appeared normal, calling into question the involvement of GR during T-cell development. These discrepancies could represent the inherent limitations of the models used. Due to early lethality in the $GR^{-/-}$ mice, only embryonic thymocytes can be examined, which may not accurately reflect the contribution of GR to T-cell maturation in the adult. In the viable $GR^{dim/dim}$ mice, the transcriptional activity of genes dependent upon GR dimerization is compromised, which

in turn could interfere with expression of, as yet to be identified, GR-induced regulatory factors that would directly (in the thymocytes) or indirectly (in other cells) influence T-cell development. Although the concentration of GR is reduced in the TKO mice, there remains residual GR in T-cells that may influence the results. Likewise, it has also been shown that the original GRKO mice express an aberrant truncated form of GR that lacks the N-terminus, but retains a hormone-binding receptor fragment that is capable of activating transcription of a subset of GR target genes (5, 18), confounding the interpretation of these studies.

More recently, T-cell GR-deficient mice were generated using an Lck promoter-driven Cre-recombinase to excise exon II of the GR gene exclusively in the thymus (referred to as TGRKO) (3). Analysis of thymocytes from these mice revealed normal cell number and subset distribution indicating that GR is dispensable for T-cell development. Polyclonal T-cell activation in TGRKO mice induced mortality due to massive gastrointestinal inflammation as a result of up-regulation of cyclooxygenase-2 (COX-2), suggesting that GR is acting, either directly or indirectly, to repress COX-2 expression (3).

To address the possibility that the effect of GR on T-cell development is stage specific, we generated independently a T-cell-specific GR-deficient mouse using Cre-recombinase driven by CD4-regulatory element (referred to as CD4-cre; $GR^{F/F}$), which removes GR at the DP stage of thymocyte development. As with the Lck-cre; $GR^{F/F}$ mice, T-cell subsets in the thymus were unchanged between the CD4-cre; $GR^{F/F}$ and control mice, indicating that GR is dispensable regardless of the stage in T-cell development at which GR is ablated. However, we found a small decrease in the number

of T-cells in the spleen of CD4-cre; $GR^{F/F}$ relative to controls. Surprisingly, activation of T-cells by anti-CD3 antibody in the CD4-cre; $GR^{F/F}$ mice did not result in inflammation of the intestine or depression of COX-2 as observed in the Lck-cre; $GR^{F/F}$ mice. Our finding suggest that GR acts early in thymocyte development, before the DP stage, to program biological events that are responsible for the activation of mature T-cells.

## *Material and Methods*

### *Gene targeting*

An 8 kb genomic DNA fragment spanning exon II of the mouse GR gene was retrieved from a 129SVJ mouse genomic library. A loxP site was inserted into the Mfe I site 420 bp upstream of exon II using a synthetic primer (5'-AATTGAGGCCTATAACT TCGTATAGCATACATTATACCGAAGTTATACGCGTC-3') containing StuI and MluI sites at the 5' and 3'ends, respectively. The modified intron carrying the loxP site and exon II were excised by digestion with EcoRV and DrdI, the DrdI site was made blunt, and this fragment was inserted into PL2-Neo plasmid cut with SmaI and SalI, the later site blunted, thus constituting the long arm. The short arm was obtained by excising the DrdI-EcoR1 site downstream of exon II. The short arm was blunted and inserted into a blunted XbaI site downstream of the floxed Neo gene.

The linearized targeting vector construct was electroporated into the 129SVJ ES cells. Approximately 1000 G418-resistant clones were isolated. Screening of the resistant clones was carried by PCR using a Neomycin and GR primer pair (pNeo forward; 5'-GGACAGGTCGGTCTTGACAAAAAGAACCG-3', and GR reverse pKO3; 5'-GCATGTTGACTTAAGTGGCTGGTGACTCAGG-3'). Putative positive clones were confirmed by Southern blot using a probe outside of the targeting construct. Two out of the eight positive clones were transiently transfected with an expression vector for Cre-recombinase to remove the Neomycin cassette. Subsequently, 500 individual subclones were cultured in duplicate in medium with or without G418. DNA from the clones that failed to grow in medium with G418 were analyzed by Southern blot using both GR and Neo probes to ensure loss of the Neomycin gene but not the floxed GR exon

II. In addition, presence of the lox site was tested by PCR using a pair of primers: F1 5'-GCTTTGGGGCATGATTTCGAACCTG-3' and B1: 5'-TTTGGATAAATCTGGCTGCGGCTGC-3', the PCR fragment was subsequently digested with StuI, a site present exclusively in the floxed allele. One positive clone was selected for injection into C57BL/6 blastocytes and resulted in germ line transmitting chimeras.

***Animal breeding and selection***

The male chimeras were bred to C57BL/6 females and the tail DNA from the progeny was genotyped using primer flanking the loxP site upstream of exon II (lox 1 forward; 5'-GGCACAGGTGAAATTGTGA-3', and lox 2 reverse; 5'-ACACATTTGGGTAAGCATGGA-3'). The heterozygous mice ($GR^{F/wt)}$ were interbred to produce homozygous mice ($GR^{F/F)}$. To generate mice with deletion of GR in the thymus, the $GR^{flox/flox}$ were bred to Lck-cre or CD4-cre mice. The resulting $GR^{F/wt}$, Lck-$cre^{+}$ or $GR^{F/wt}$, CD4-$cre^{+}$ were interbred to produce the littermates $GR^{F/F}$, cre+ mice or $GR^{wt/wt}$, cre+ mice that we used as controls in all our experiments. The primers for genotyping Lck-cre mice are: Lck forward; 5'-CCTCCTGTAACTTGGTGCTTGAG-3' and Lck reverse; 5'-TGCATCGACCGGTAATGCAG-3'. The primers for genotyping CD4-cre mice are: CD4 forward; 5'-TCTCTGTGGCTGGCAGTTTCTCCA-3', and CD4 reverse; 5'-TCAAGGCCAGACTAGGCTGCCTAT-3'. PCR reactions were performed in 20 μl reaction using 1 unit of Taq DNA polymerase with buffer A (Fisher Scientific), in presence of 0.2 mM of dNTP mix, and 500 nM of each primer. The reaction was

denatured 5 min at 95°C, then underwent 30 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C.

All mouse protocols were in accordance with National Institute of Health guidelines and were approved by the Animal Care and Use Committee of NYU School of Medicine (New York, NY).

***In vivo antibody treatment***

Mice were injected intraperitoneally with a 100 μg or 200 μg of anti- CD3e antibody (145-2C11) diluted in 250 μl PBS and analyzed 16 hours post-injection.

***Flow cytometry***

Thymocytes were harvested from 6-week old male mice and 2-10x$10^6$ cells were incubated with anti-CD16/CD32 (2.4G2) antibody for 15 min on ice and then stained with combination of antibodies against the following cell surface proteins: anti-CD4-APC (GK1.5), anti-CD4-APC-Cy7 (GK1.5), anti-CD8α-PE-Cy7 (53-6.7), at appropriate concentrations for 30 min on ice. The cells were then washed twice with PBS and analyzed on a BD-LSRII flow cytometer (Becton Dickinson, Mountain View, CA). For the apoptosis experiments, isolated thymocytes were treated with either $10^{-6}$ M Dexamethasone or an equal volume of the ethanol vehicle in tissue culture media (RPMI-1640 with 5% FCS, 2mM Glutamine, penicillin/streptomycin 5 Units/ml) for 24h at 37°C, 5% $CO_2$. Annexin-V staining was performed following the protocol provided by the manufacturer (BD PharMingen, San Diego, CA). After two washes with cytoperm/cytowash, the cells were re-suspended in FACS buffer and analyzed by flow

cytometry. All data were analyzed using Flowjo flow cytometric data analysis software (Tree Star, Ashland, OR). Statistical analysis of T-cell populations was performed using T-tests. Statistical significance was considered for P-values < 0.05.

***Protein analysis***

Whole cell protein lysates were prepared from whole thymus, isolated thymic $CD4^+$ or $CD8^+$ T-cells, or splenic T-cells sorted using CD90 (Thy 1.2) microbeads and the MACS separation columns (Miltenyi biotec). Cells were lysed in 0.1 ml of buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol, and additional protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 8 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The lysates were centrifuged at 12,000 rpm for 15 min at $4^oC$. The soluble supernatants were normalized for total protein concentration using the Bio-Rad protein assay. Samples were boiled for 3 min in 1 X SDS sample buffer and stored at $-20^oC$.

For Western blotting, 30 μg of protein extracts were resolved on a 10% SDS-PAGE, transferred unto PVDF membranes and probed with antibodies to the GR N-terminal (M20; Santa-Cruz Biotechnology) or C-terminus (P-20; Santa-Cruz Biotechnology), mouse monoclonal COX-2 antibody (5E10/D10; abcam), mouse monoclonal antibody against tubulin (TU27; Covance) and a polyclonal antibody against hsp90 (Santa-Cruz Biotechnology) were used as controls for loading.

***Quantitative Real time PCR***

Total RNA from purified T-cells cells was extracted with Trizol (Invitrogen) as described by the manufacturer. cDNA was synthesized using the Enhanced Avian Reverse Transcriptase (Sigma) and random primer hexamers following the manufacturer's instructions. cDNAs were amplified using the SYBR Green Quantitative PCR Kit (Sigma) on a LightCycler (Roche). Reactions were carried out in a 20 µl reaction containing a 500 nM concentration of each primer and the SYBR Green *Taq* ReadyMix for Quantitative RT-PCR (Sigma) as recommended by the manufacturer with the following conditions: 95°C for 2 min, followed by 42 cycles of 5 sec at 95°C, 5 sec at 55 °C and 10 sec at 72°C. COX-2, mRNA levels were normalized to 28S expression. All RT-PCR products were analyzed in a post-amplification fusion curve to ensure that a single amplicon was obtained. Primers used were: COX-2-forward- (5'-ATCCCCCCACAGTCAAAGACA-3'); COX-2-reverse, (5'-CATACATCATCAGACCAGGCACC-3'); GR-forward (5 -CCTAAGGACGGTCTGAAGAGC-3); GR-reverse, (5 -GCCAAGTCTTGGCCCTCTAT-3); 28 S rRNA-forward (5'-AAACTCTGGTGGAGGTCCGT-3') and 28 S rRNA-reverse (5'-CTTACCAAAAGTGGCCCACTA-3').

***Histology***

Tissues were fixed in 10% neutral buffered formalin overnight, washed in 70% ethanol and embedded in paraffin. Hematoxylin and eosin staining was performed on 5 µm-wide sections. Micrographs were captured on an Axioplan 2 Zeiss microscope.

***Results***

T-cell glucocorticoid receptor (GR)-deficient mice were generated using CD4 promoter-driven, Cre recombinase-mediated excision of the LoxP-flanked (floxed) exon II of the GR gene (Figure 1). Genotyping of the mice was carried out by PCR and confirmed by Southern Blot analysis (Figure 2A-C). The mice homozygous for the floxed GR gene and harboring the CD4-cre transgene (designated CD4-cre;$GR^{F/F}$) were as healthy as their CD4-Cre negative homozygous floxed GR littermates. Efficient excision of exon II in the thymus was confirmed by Southern blot (Figure 2D). In addition, we found little GR mRNA (Figure 2E) and protein (Figures 2F and 2H) in the thymus. No GR was detected in purified $CD4^+$ and $CD8^+$ single positive thymocytes from CD4-cre;$GR^{F/F}$ mice, indicating the efficient loss of GR at the specified stage (Figure 2G). Detection of GR protein was carried out using either an antibody to the GR amino-terminus, recognizing an epitope in exon II, or an antibody to the GR carboxy-terminus, recognizing an epitope outside the deleted exon. In both cases, intact GR was not detected and no specific bands were evident in thymocytes from CD4-cre;$GR^{F/F}$ relative to wild type control mice, excluding the possibility of a remaining C-terminal truncated form of GR in our mouse model (Figure 2H). GR expression remained intact in the lung (Figure 2G), and other tissues (not shown) where CD4-cre would not be expressed. Therefore, GR expression is efficiently and specifically ablated in thymocytes in CD4-cre;$GR^{F/F}$ mice.

We next analyzed developing thymocytes and peripheral T-cells in the mutant mice. We noted no significant difference in the cell number or subset distribution in the thymus between genotypes (Figure 3 and Supplemental Figure 1). Peripheral T cell

populations and subsets were also not significantly different in Peyer's Patchs and inguinal lymph nodes between the CD4-cre;$GR^{F/F}$ mice and CD4-cre;$GR^{wt/wt}$ controls (Supplemental Figure 2). In contrast, in the spleen we observed a small but significant decrease (~40%) in both $CD4^+CD8^-$ and $CD4^-CD8^+$ cell populations (Figure 3 and Supplemental Figure 1). Thus, GR deficiency at the $CD4^+CD8^+$ stage is not required for thymocyte development, whereas GR may have a small effect on the trafficking or survival of mature T-cells in the spleen.

We also monitored T-cell apoptosis *in vitro* in response to Dexamethasone treatment by Annexin-V staining. When treated with Dexamethasone, $CD4^+CD8^+$ and $CD4^+CD8^-$ T-cells from control mice expressing GR underwent apoptosis, whereas thymocytes from CD4-cre;$GR^{F/F}$ mice were largely resistant to glucocorticoid-mediated cell death (not shown). This is in agreement with the established role of GR in thymocyte apoptosis.

T-cell receptor activation induces the expression of a host of proinflammatory mediators, including TNF-alpha, iNOS and COX-2 (10, 11). A previous study in mice in which GR had been removed at the early double negative $CD4^-CD8^-$ stage of T-cell development by Lck-Cre (Lck-cre;$GR^{F/F}$), showed a profound derepression of COX-2 mRNA and intestinal inflammation upon anti-CD3 induced T-cell activation (3).

To determine the proinflammatory effect of GR-deficiency in activated T-cells in our mouse model, we examined COX-2 expression after administering anti-CD3 specific antibody to CD4-cre;$GR^{F/F}$ and control mice. Surprisingly, administration of anti-CD3 antibody (either a single dose, a double dose or two consecutive doses) to the CD4-cre;$GR^{F/F}$ mouse did not result in lethality in contrast to what was described for the Lck-

cre;$GR^{F/F}$ (3) and showed no signs of illness (feeding and mobility) compared to the $GR^{wt/wt}$ and CD4-cre;$GR^{wt/wt}$ controls (not shown). Although COX-2 mRNA (Figure 4B;inset) and protein levels (supplementary Figure 3) were induced in purified T-cells after treatment with antibody to CD3 in CD4-cre;$GR^{F/F}$ and control mice, there was no difference in the regulation of COX-2 between CD4-cre;$GR^{F/F}$ and controls (CD4-cre;$GR^{wt/wt}$) despite the lack of GR mRNA and protein. This finding indicates that deregulation of COX-2 in T-cells in the absence of GR is thymocyte stage-specific and is required only during early thymocyte development.

To ensure that the differences in response in GR-deficient T-cells between Lck-cre;$GR^{F/F}$ and CD4-cre;$GR^{F/F}$ were stage specific rather than a result of variation in the GR targeted mouse strain between groups, we generated our own Lck-driven thymus-specific GR knock out mouse using the same GR conditional allele as in the CD4-cre;$GR^{F/F}$. We examined the mice from control, Lck-cre;$GR^{F/F}$ and CD4-cre;$GR^{F/F}$ by histology for signs of intestinal inflammation after T-cell activation by anti-CD3 administration and for effects on COX-2 mRNA expression. In agreement with a previous report, the Lck-cre;$GR^{F/F}$ mice displayed intestinal inflammation, with regions of the epithelium showing an infiltration of small lymphocytic cells (Figure 4 f-h) that was associated with the up regulation of COX-2 mRNA upon polyclonal T-cell activation (Figure 4B) (3). In contrast, CD4-cre;$GR^{F/F}$ mice had normal intestinal morphology (Fig 5; panels a-d) and, like the control mice, showed no signs of gastrointestinal inflammation and no COX-2 overexpression in response to T-cell activation relative to control (Figure 4B). Other inflammatory gene products, including TNF-alpha and iNOS did not display this up regulation when GR was removed at different stages of thymocyte

development (Figure 5A and B). Thus, removal of GR early in thymocyte (Lck-cre;$GR^{F/F}$) development results in gastrointestinal inflammation when mature T-cells are activated. Surprisingly, this effect is not observed when GR is removed later in thymocyte development (CD4-cre;$GR^{F/F}$). Therefore, GR imparts a memory to thymocytes early in development that persists in mature T-cell stages to curb COX-2 expression.

***Discussion***

The role GR plays during thymocyte differentiation as well as its subsequent effects in adult T-cell lineages has not been fully elucidated (2). An important study from the Muglia laboratory addressed the role of GR in T-cell activation in adult mice by generating a conditional GR knockout mouse and ablating GR at the early $CD4^{-}CD8^{-}$ stage of thymocyte development using Lck-Cre recombinase. T-cell GR deficiency resulted in a profound up-regulation of COX-2 mRNA, gastrointestinal inflammation and death upon polyclonal T-cell activation (3). Our results also show that GR-deficient Lck-cre;$GR^{F/F}$ mice develop intestinal inflammation as a consequence of T-cell activation and elevated COX-2 mRNA expression.

While GR-deficiency in early T-cell development (Lck-cre;$GR^{F/F}$) leads to inflammation and pathology, the CD4-cre;$GR^{F/F}$ animals display, interestingly, no signs of gastrointestinal inflammation or COX-2 overexpression relative to control mice when T-cells are activated by anti-CD3 antibody. The striking stage specific phenotypic consequences of the loss GR suggest that, early in T-cell development, GR institutes a genetic program that persists throughout development and, once established, GR is then dispensable. This pattern may be established by GR activation in thymocytes through the paracrine action of glucocorticoids produced locally by the thymic epithelium.

In addition to the traditional role of GR as a transcriptional regulatory protein affecting gene specific expression, GR also participates in the epigenetic regulation of gene expression (9). Epigenetic changes in gene expression occur through alterations in histone modification that affect chromatin structure or through chemical modifications of the DNA, such as methylation (12). GR has been shown to promote local chromatin

remodeling and changes in histone modification (6), in addition to altering the DNA methylation pattern at particular promoters (16, 29). It is well established that the differentiation of immature thymocytes into mature T-cells requires the activation and silencing of multiple genes (27, 28), and it is conceivable that GR influences gene expression in a stage specific manner in T-cell development through both genetic and epigenetic events.

Our findings suggest a model whereby early in thymocyte development GR is activated by glucocorticoids emanating from the thymic epithelium, which induces epigenetic alterations, such as DNA methylation or histone modifications, resulting in the decreased transcription of proinflammatory genes (Figure 6). If GR were removed early in thymic development, then this epigenetic suppression would not occur. However, if GR were removed at a later point in thymocyte development, then the repression of promoters by GR would have already been established. According to this model, subsequent hyperinduction of proinflammatory mediators, such as COX-2, upon T-cell activation would be observed only if GR were removed early in development, and is consistent with the derepression of COX-2 when GR is ablated at the beginning of thymocyte maturation.

Indeed, the COX-2 upstream regulatory region harbors CpG islands and undergoes DNA methylation. Suppression of this methylation by inhibitors, such as 5-azacytidine, results in the hyperinduction of COX-2 upon stimulation, presumably by enabling transcription factors, normally excluded, to bind and activate the promoter (26). Thus, GR may induce a particular epigenetic state early in thymocyte development, establishing a threshold for the activation of proinflammatory genes. This proposed

epigenetic GR anti-inflammatory mechanism is novel and distinct from the traditional GR anti-inflammatory mechanism by direct repression of gene expression through protein-protein interaction. Perturbations of GR-regulated events in early in thymocyte development could lead to the enhanced expression of proinflammatory mediators by activated T-cells, and contribute to the etiology of T-cell-dependent inflammatory diseases such as lupus, psoriasis and Crohn's disease.

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*Figure Legends*

**Figure 1 Generation of a floxed conditional allele of *GR* (*GR*flox).**

(*A*) Schematic representations of a) the wild-type *GR* locus flanking exon II, b) targeting vector, c) recombined locus, and d) the final conditional allele of *GR*(*GR* flox) with the PGK-neo selection cassette removed are shown. *B*) Confirmation of the *GR*flox allele by Southern blot. DNA prepared from ES cell clones was digested Bcl1 and Southern-blotted for hybridization with the 3' and neo probes indicated in *A*. C) Presence of the loxP site in the *GR*flox allele was confirmed by PCR. DNA from ES cells was amplified by PCR using F1 and B1 primers. The PCR products, either uncut (-) or digested with Stu I (+) were resolved on an agarose gel and visualized by ethidium bromide staining.

**Figure 2 Deletion of GR in T-cells of *GR*flox mice.**

(*A*) Schematic diagram of the *GR*flox conditional allele. B) Southern analysis of *GR*flox mice. DNA from wild type (W/W), heterozygous (F/W) or homozygous *GR*flox ($GR^{F/F}$) mice was digested with StuI and Southern-blotted for hybridization with the 3' end probe indicated in A. B) Genotyping of *GR*flox mice by PCR. DNA was amplified by PCR using Lox1 and Lox2 primers and the resulting DNA fragments were resolved on an agarose gel and visualized by ethidium bromide staining. D) DNA prepared from the thymus of mice were digested with Bcl1 and Southern-blotted using a 3' probe. (E) mRNA abundance of GR in $CD4^+$ or $CD8^+$ T-cells was assessed by qPCR using 28S RNA as the normalization control. F) Western blot analysis of protein extracted from the whole thymus using an antibody against GR and tubulin. G) Western blot analysis of protein extracted from purified $CD4^+CD8^+$ T-cells or lungs from mice of the indicated

genotype using an antibody against GR and tubulin. H) GR protein immunoblot of whole thymus using antibodies to the GR N-terminus (*left panel*) and C-terminus (*right panel*). A similar non-specific pattern of bands is observed in both CD4/GR$^{wt/wt}$ (WT) and CD4/GR $^{f/f}$ (Flox) samples.

**Figure 3 Distribution of T-cell subsets in CD4-cre;GR$^{F/F}$ thymus and spleen**

Thymus and spleen were removed from 6-7 week old CD4-cre;GR$^{wt/wt}$ (WT) and CD4-cre;GR$^{F/F}$ (F/F) mice. T-cells were isolated and analyzed by flow cytometry as described in the Materials and Methods. *n=4, $p<0.005$

**Figure 4 Differential inflammatory responses in T-cell deficient mice.**

A) Histological analysis of ceca from CD4-cre;GR$^{wt/wt}$ (Control), CD4-cre;GR $^{F/F}$ and Lck-cre;GR$^{F/F}$ mice were treated with PBS or an antibody to CD3 (anti-CD3). Sections are representative of *n* = 3 mice. Magnification: 100X panels a-f; 400X panels g-h

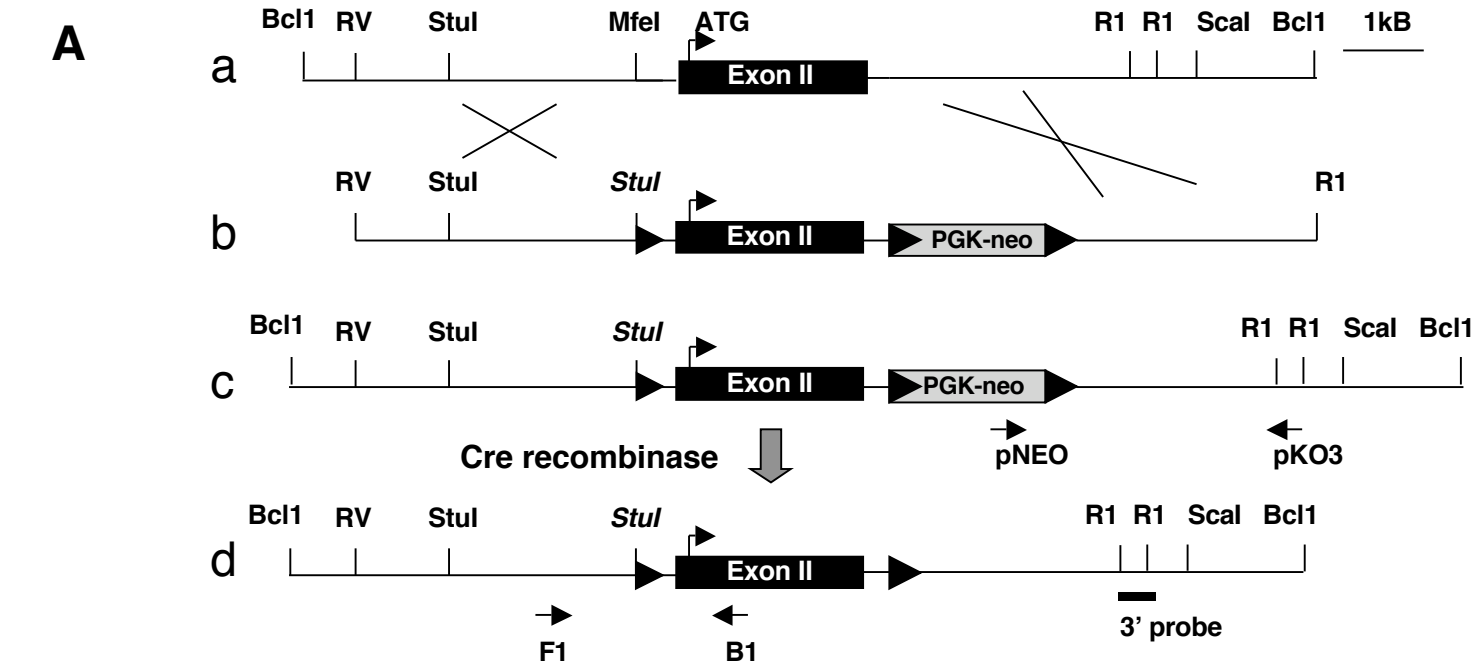
B) COX-2 expression in T-cells**.** COX-2 mRNA abundance in purified T-cells from control (CD4-cre;GR$^{wt/wt}$) and CD4-cre;GR$^{F/F}$ and Lck-cre;GR$^{F/F}$ mice after injection with PBS (-) or antibody to CD3 was assessed by qPCR using 28S RNA as the normalization control. The CD4-cre;GR$^{wt/wt}$ PBS control samples were arbitrarily set as 1. Samples were run in triplicate and the error bars represent SD. C) Western blot analysis of protein extracted from purified T-cells from the indicated genotypes using an antibody against GR and hsp90. The experiment was repeated three times with similar results.

**Figure 5 Inflammatory gene responses in T-cell deficient mice.** TNF alpha and iNOS mRNA abundance in $CD4^+$ $CD8^+$ T-cells from the indicated genotype was assessed by qPCR using 28S RNA as the normalization control. The CD4-cre;$GR^{wt/wt}$ PBS control samples were arbitrarily set as 1. Samples were run in triplicate and the error bars represent SD.

**Figure 6 Model for the stage specific regulation of gene expression by GR in thymocytes**

Schematic representation of a thymic lobule and thymocyte developmental pathway. Glucocorticoid hormone (black circle) is produced by the thymic epithelium and activates GR (gray square). The receptor in turn affects the overall program of gene expression by epigenetic changes (depicted by a ball and stick) in DNA methylation or histone modification of the regulatory regions of certain proinflammatory target genes. This change is maintained in the mature T-cells in the periphery. Upon T-cell receptor activation (wiggly arrow) in WT and Lck-cre;$GR^{F/F}$, epigenetic imprinting is already established, such that only a single transcription factor (parallelogram) binds to the promoter and activates the expression of this pro-inflammatory gene to the appropriate level. When epigenetic imprinting is lost, as in the Lck-Cre;$GR^{F/F}$, then additional transcription factors are capable of binding (stripped oval) and hyperactivating the target gene upon TCR signaling, leading to overexpression of proinflammatory mediators, such as COX-2.

# Figure 1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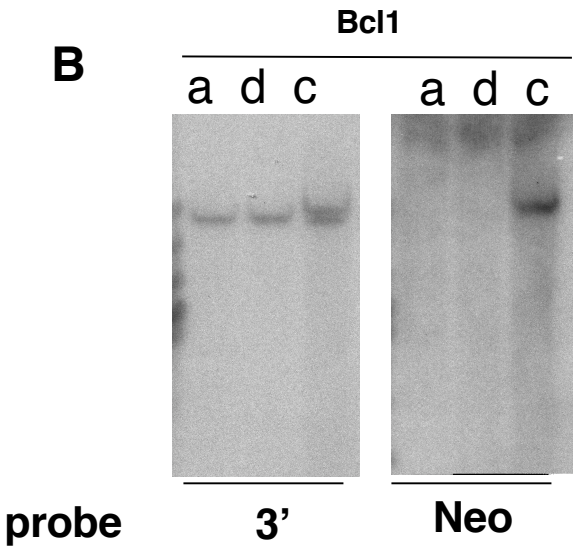
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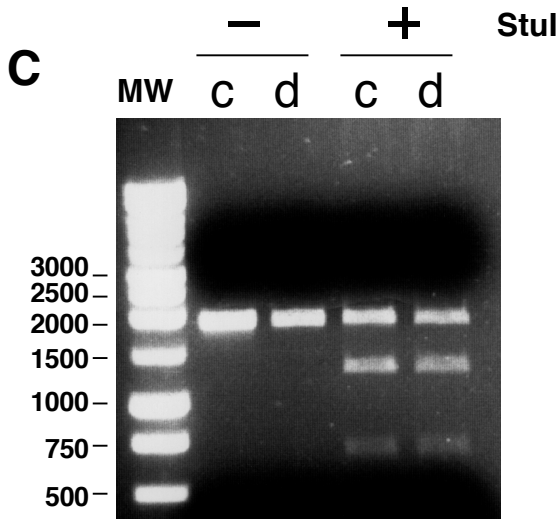
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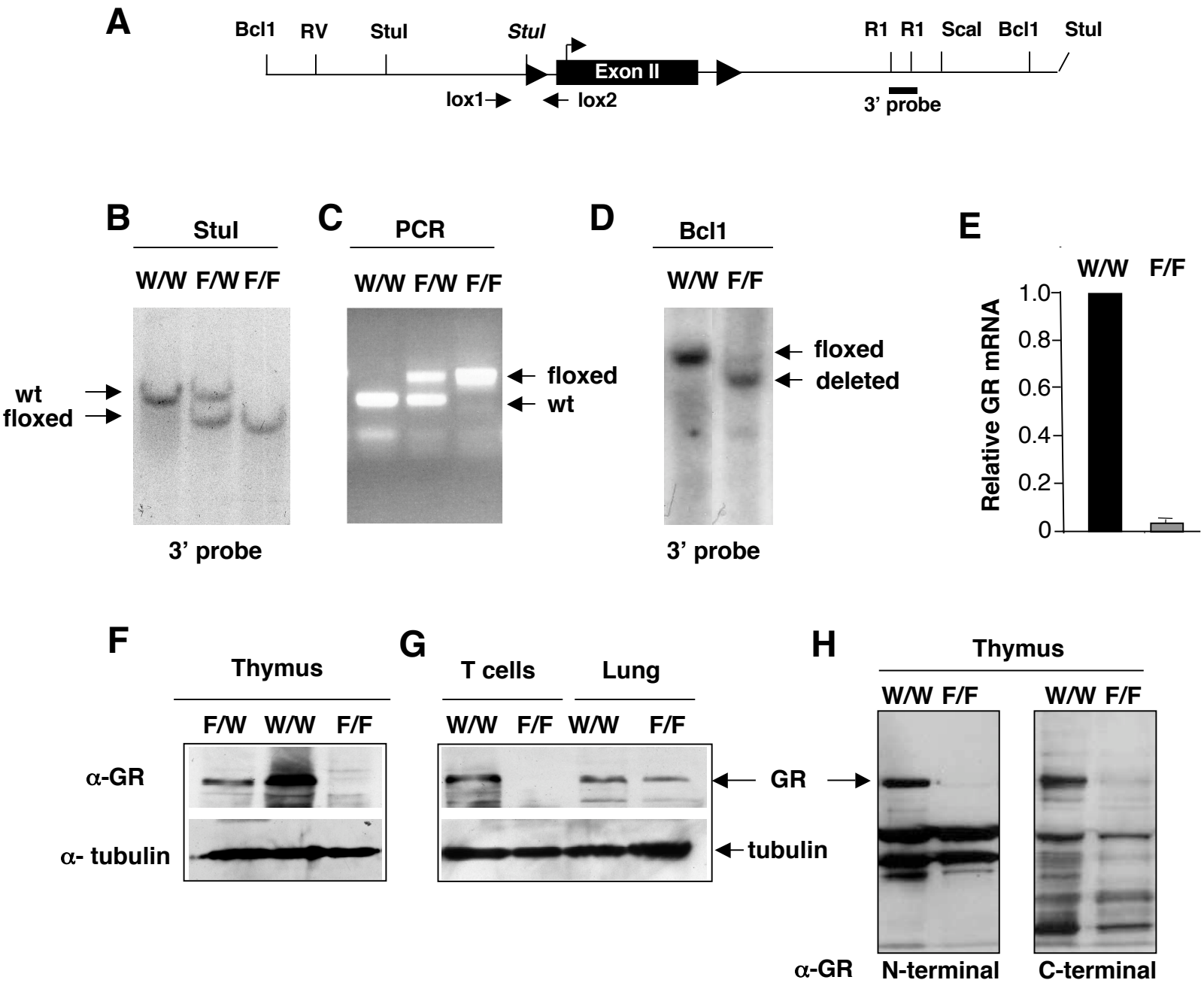
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wt
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W/W F/F
Relative GR mRNA
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0.8
0.6
0.4
0.2
0
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α-GR
α- tubulin
G
T cells
Lung
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N-terminal
C-terminal

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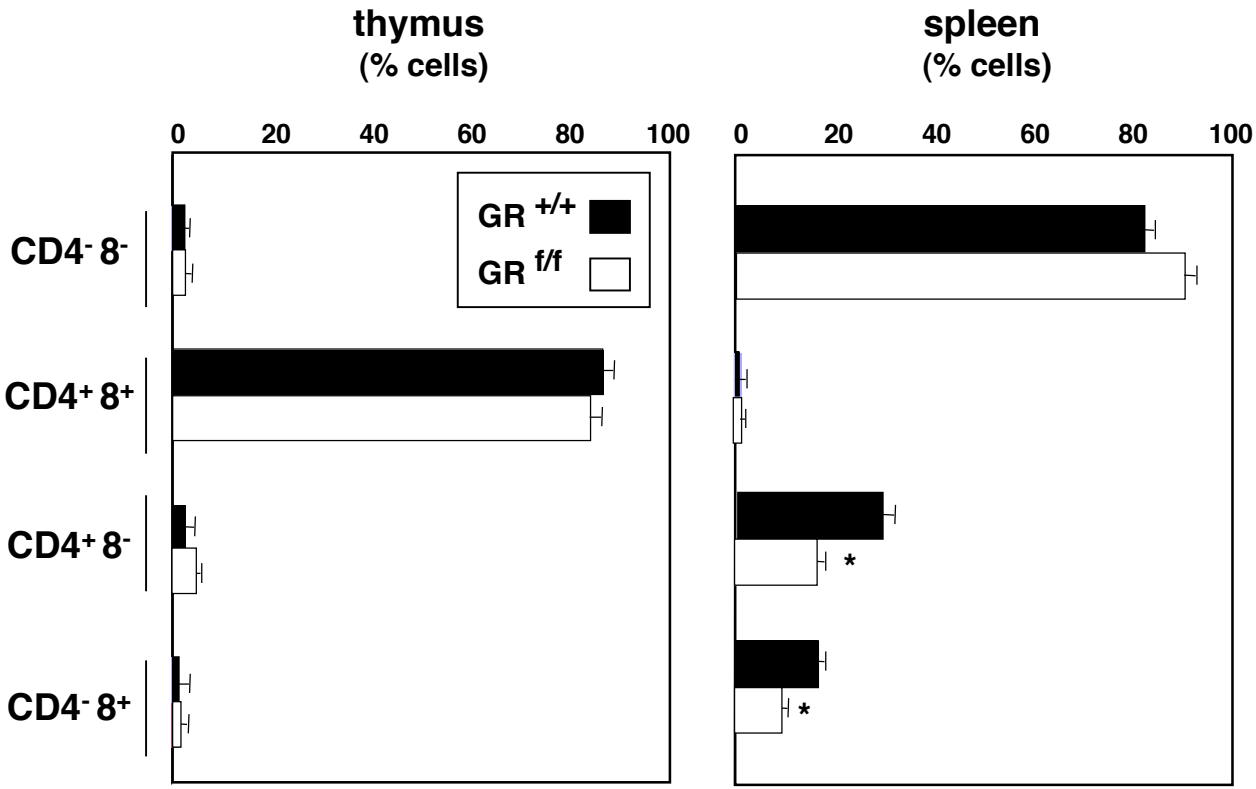
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(% cells)
spleen
(% cells)
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CD4+ 8+
CD4+ 8-
CD4- 8+
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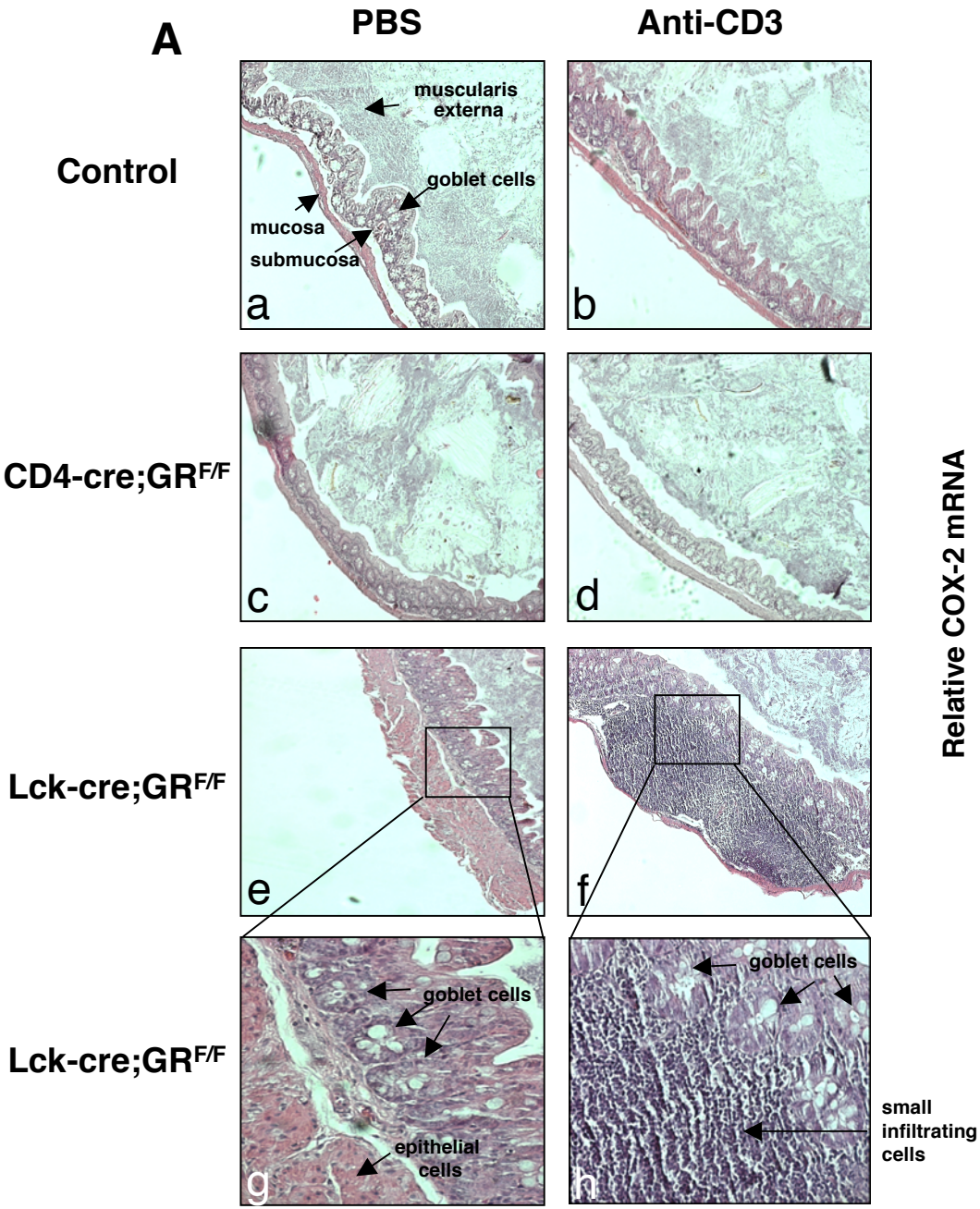
# Figure 4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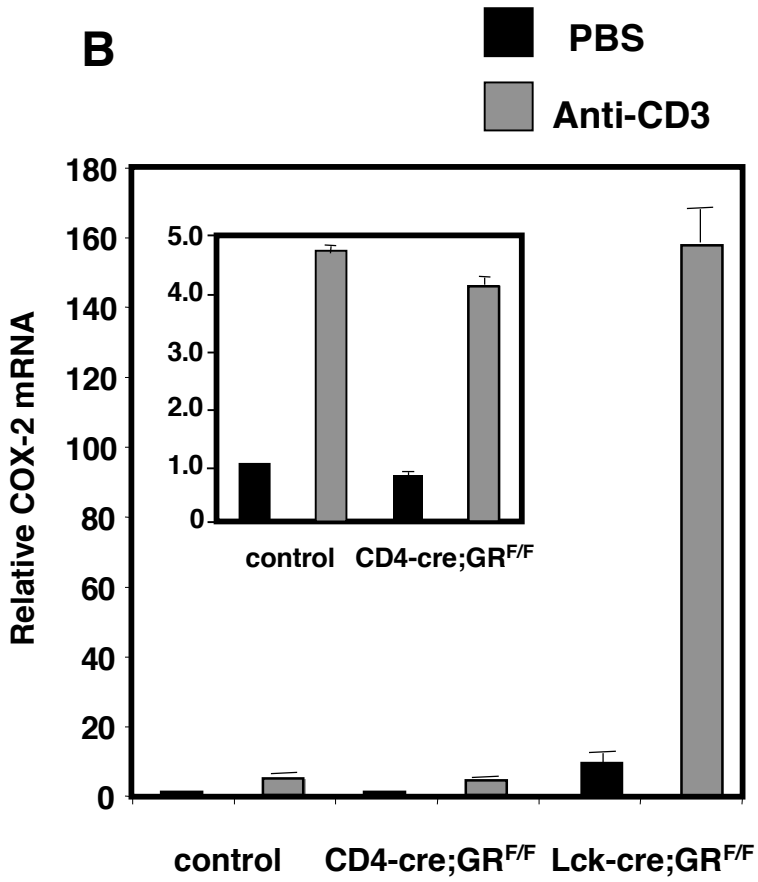
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3.0
2.0
1.0
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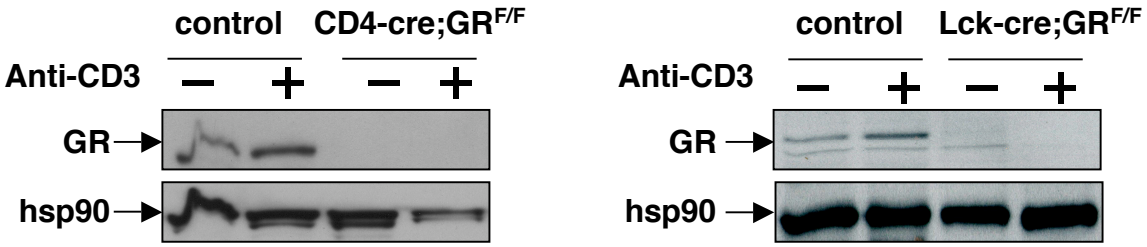
Lck-cre;GR^F/F

# Figure 4

**C**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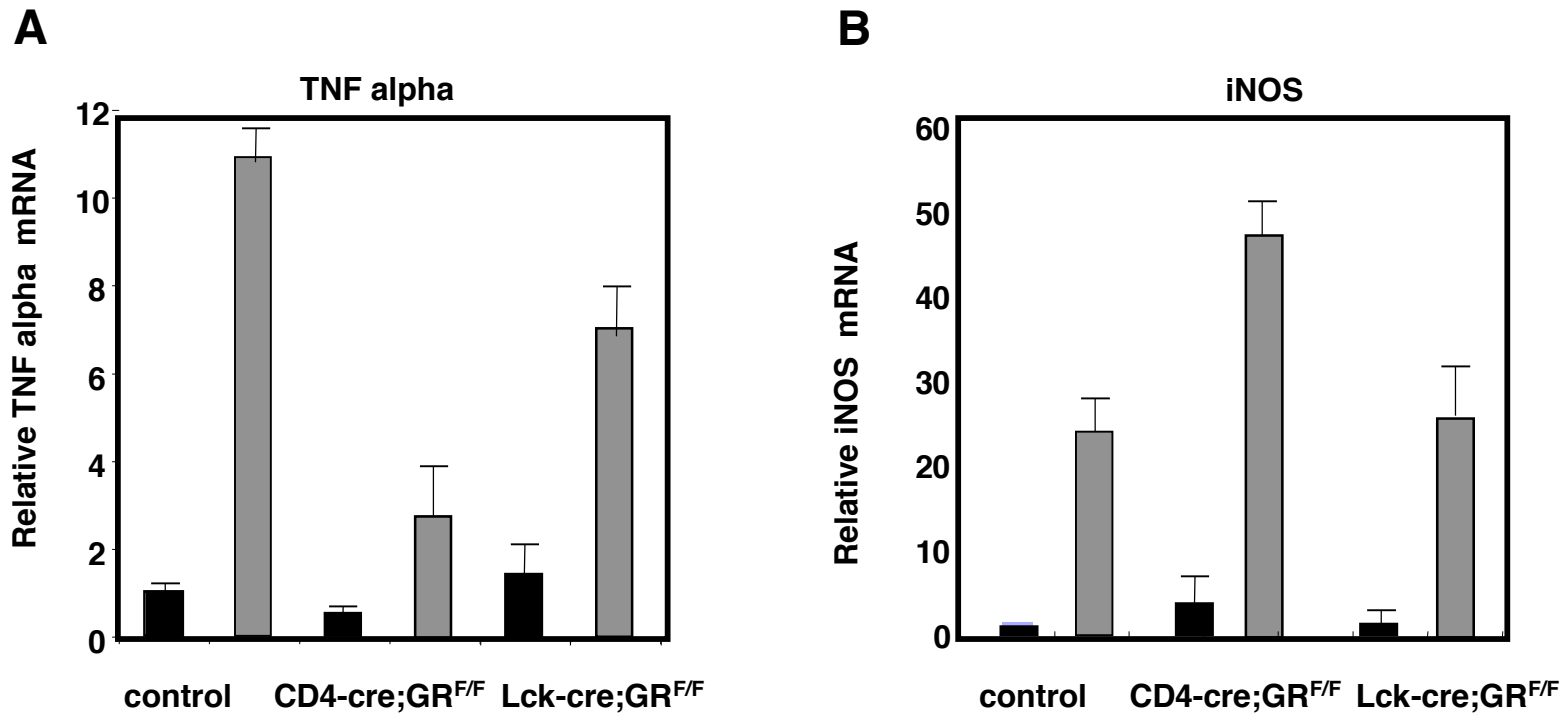
GR
hsp90

# Figure 5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B
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20
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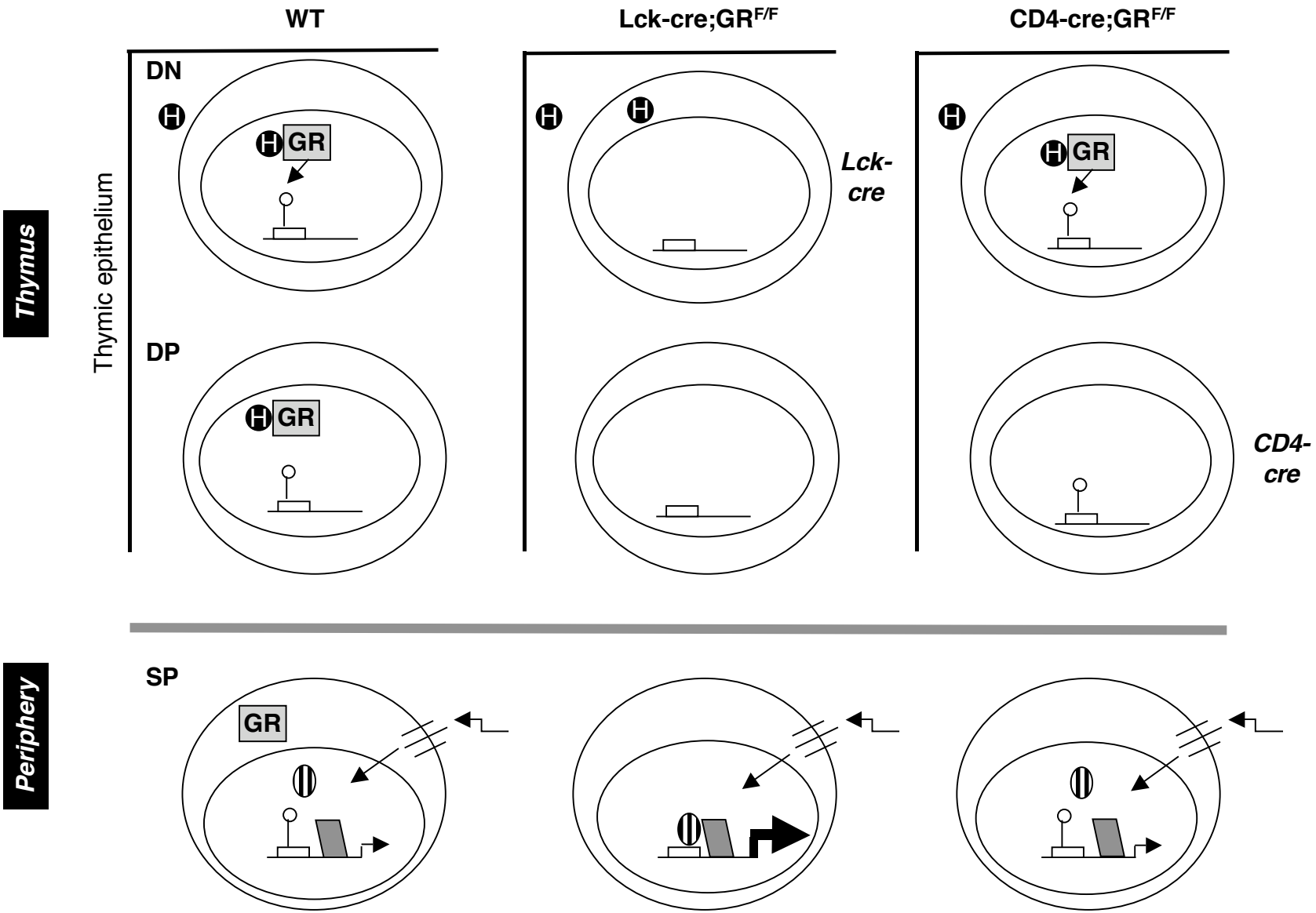
# Figure 6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CD4-cre;GR F/F
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DP
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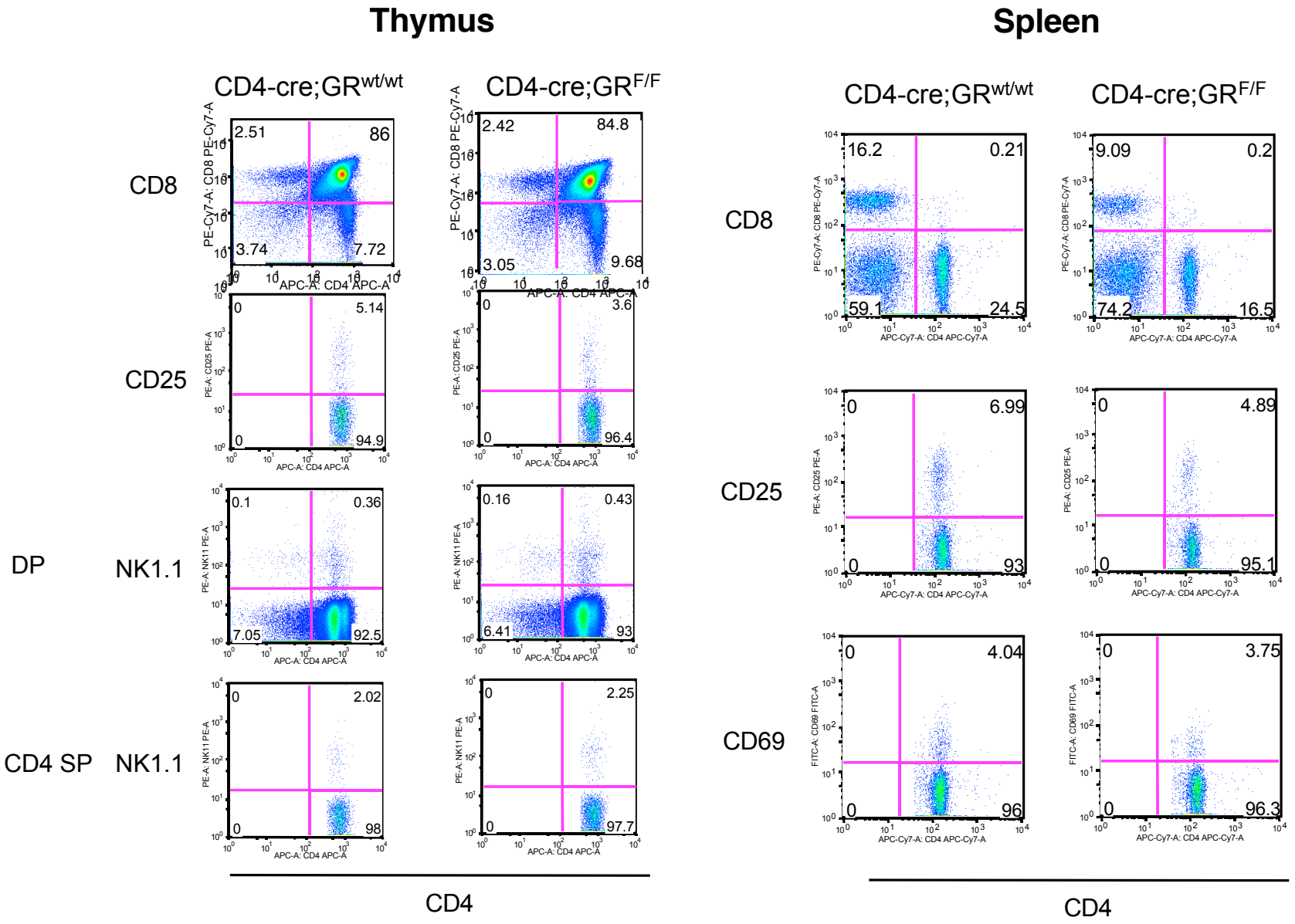
GR
Lck-cre
CD4-cre
Periphery
SP

# Supplemental Figure 1



# Supplemental Figure 2